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Original Article

FORMULATION AND EVALUATION OF PROBIOTIC AND PREBIOTIC LOADED PELLETS BY EXTRUSION AND SPHERONIZATION FOR IMPROVED STORAGE VIABILITY

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ABSTRACT

Objective: The present study aims to prepare stains-loaded enteric-coated pellets by extrusion and spheronization technique for acidic environment protection and improve the viability of the strains during storage.

Methods: *Lactobacillus casei* and *Lactobacillus plantarum* strains are proven to have various therapeutic and prophylactic uses in human beings, but low stability during storage and transit to site of action has limited their action. Pellets were prepared by incorporating probiotic strains D1-D9 (*L. casei*) and E1-E9 (*L. plantarum*) by further enteric-coating the pellets, which were evaluated for particle size, loss on drying, friability, micromeritic properties, viability, disintegration, survivability in acidic and bile juices, and stability studies for 90 d respectively.

Results: The method employed for preparing the pellets showed good % yields with a particle rage of 1400-850 µm. LoD values were in the range of 3.07±0.30% to 2.13±0.11%; all the prepared pellets showed good flow properties and friability in an acceptable range. SEM images revealed that enteric-coated pellets had smooth and uniformly surfaces. The viability results ranged from 8.78±0.31 to 8.53±0.15 log CFU/g and 8.47±0.15 to 8.85±0.22 log CFU/g for both *L. casei* and *L. plantarum* enteric coated pellets, respectively. The Disintegration time for the pellets was<15 min in all the formulations. The enteric-coated probiotic pellets provided adequate protection against the acidic environment. Studies of survivability in simulated gastrointestinal conditions demonstrated that formulations D7 and E7 showed higher viability among the formulations at the end of 3 h. The stability studies showed that the formulations with a higher concentration of Inulin and pectin combination proved better viability of *L. casei* and *L. plantarum* strains in the formulation during 90 d of stability study.

Conclusion: This study suggested that using extrusion and spheronization techniques can be employed to prepare pellets with prebiotics (Inulin and Pectin) which can be utilized to formulate probiotic dosage forms with improved viability in physiological conditions and real-time storage conditions.

Keywords: Probiotics, Prebiotics, Inulin, Avicel, Pectin, Pellets

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INTRODUCTION

Probiotic originated from Greek terminology where 'pro' denotes "promoting" while 'biotic' denotes "life". As per the definition embraced by WHO, probiotics are: "live microorganisms which when administered in adequate amounts confer a health benefit on the host". Probiotic bacteria are live, non-pathogenic microorganisms that have improved the host's health by restoring natural gastric flora [1].

Lactobacillus casei (*L. casei*) and *Lactobacillus plantarum* (*L. plantarum*) are proven and safe probiotic strains which have various therapeutic and prophylactic uses in human beings. they are also used to minimize the duration/occurrence of respiratory infections [2], improve gut health by altering the microflora balance in the gut, improve digestion, modulate immune responses in general, which benefit patients suffering from allergies, and cancer [3]. *Lactobacillus plantarum* has proven to be beneficial in the treatment of increased blood pressure [4], elevated cholesterol levels, diabetes [5] and Anxiety [6]. Marcos A *et al.*, have claimed that *Lactobacillus casei* strains are also able to control lymphocytes and CD56 cells caused due to stress of academic examinations [7]. Prebiotics are generally non-digestible supplements that benefit the host's health by selectively promoting the proliferation and functioning of specific genera of colonic microbes, typically lactobacilli and Bifidobacteria [8].

A probiotic must pass through the harsh manufacturing process and then the gastric environment as a pharmaceutical preparation. According to World Health Organization (WHO) and Food and Agriculture Organization (FAO), the minimal viability of probiotics in a product should be 10^6 CFU/g throughout its shelf life, considered a minimum therapeutic level [9]. Probiotics are available in several dosage forms: pills, capsules, suppositories, frozen dried powders, frozen granules, juices, and fermented dairy products; however, the incorporation of probiotics into pellets by extrusion and spheronization [10] has attracted many formulators because of its simple process, reproducible results, better yields, and scalability [11, 12]. Pellets can maintain the viability of probiotics and deliver them to their site of action [12].

Avicel® PH-101 was used as the pelletizing agent as it has been proven to have the optimum extrudable characteristics for the wet mass [13]; however, it possesses a drawback of not disintegrating completely; hence, to overcome this issue a super disintegrant and a binder Polyvinyl pyrrolidone [14] was used in a concentration of 5% in all the formulations. Inulin and Pectin [15–17] have been used in various formulations of probiotic strains [18] to improve their viability during the transition to GIT and storage conditions [19]. In October 2013, the International Scientific expert team of the International Scientific Association for Probiotics and Prebiotics (ISAPP) reviewed the concept, thereby renewing the definition of "synbiotic" in 2019 as "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host"[20].

Hence, the study focuses on incorporating probiotic strains *L. casei* and *L. plantarum* individually along with Inulin and Pectin as prebiotics in pellets by extrusion and spheronization technique, further enteric coat the pellets and study the impact of enteric coating and prebiotics in maintaining the viability of the probiotics during the manufacturing and storage conditions.

MATERIALS AND METHODS

Materials

Inulin and Pectin were procured from Merck Specialties Pvt Ltd, India. Avicel® PH-101 was a gift from Steer life Pharma, India. Kollicoat® MAE 30DP, Pancreatin, Pepsin and Skim milk were procured from Sigma Aldrich, India. Polyvinylpolypyrrolidone was purchased from Loba Chemie Private Limited, India. *L casei,* ATCC® 393™, *L plantarum* ATCC®8014™strains, Agar Powder and De Man, Rogosa and Sharpe (MRS) agar were purchased from HIMEDIA Laboratories India, the probiotic strains were maintained at-20 °C till use.

Methods

Pre-formulation studies

Fourier transform infrared spectroscopy (FT-IR)

The compatibility of individual excipients (Inulin, Pectin, Avicel® PH-101, Kollicoat) and Physical mixture was studied using Shimadzu FTIR 8400S. The samples were made by pressing the mixture of excipients with potassium bromide and scanned in the wavenumber range of 4,000 to 400 cm^{-1}. The spectra obtained from FTIR analysis were compared for compatibility between the excipients used.

Differential scanning calorimetry (DSC)

The DSC of individual excipients (Inulin, Pectin, Avicel® PH-101, Skim milk) and physical mixtures of excipients were studied using Shimadzu-thermal analyser DSC-60, Japan. Under the nitrogen environment, at a calorimetric scan rate of 10 °C/min and scan was conducted in the range of 30-350 °C.

Cultivation of probiotic strains

The procured freeze-dried *Lactobacillus casei* ATCC® 393™ and *Lactobacillus plantarum* ATCC®8014™ sticks were transferred to previously prepared sterile *Lactobacillus* MRS broth, separately and were incubated at a temperature of 37 °C for a period of 24 h in a bacteriological incubator. The grown culture was compared with the control [21].

Subculture was transferred to a centrifuge tube under LAF conditions, and the centrifugation was done at the speed of 4000 RPM for a period of 15 min at 4 °C. The supernatant liquid was removed, and the cell pellet of *L. casei* and *L. plantarum* was washed with saline solution twice. These cells were used for mass cultivation of the probiotic strains with MRS broth at the time of preparing the granulating mass for extrusion and spheronization.

Preparation of pellets by extrusion and spheronization technique

Preparation of direct inoculated probiotic pellets of L. *casei and L. plantarum* using Avicel® PH-101

Avicel® PH-101 and other excipients were passed through sieve #40 to avoid any clumps formation. The powders were blended using a concentration of 10 log CFU/ml *L. casei* and *L. plantarum* strain dispersion in Skim milk (2%) separately and water as granulating fluid with the help of a rapid mixer granulator to produce a wet mass of extrudable consistency. The powders were mixed in a specific ratio as mentioned in table 1. The process parameters were selected as per the optimized conditions suggested by DoE trials. This mass was transferred to the 1.0 mm pore size extruder and was made to rotate at 31RPM speed until rod-shaped extrudates were obtained. These extrudates were immediately transferred to a spheronizer. The spheronizer was made to rotate at 1350 RPM speed until pellets of perfect spheres were obtained. The pellets were dried using a hot air oven for 2-3 h at 40 °C.

Experimental design

Screening and optimization of process parameters for the preparation of direct inoculated pellets

For screening and optimizing the process parameters 2^3 factorial design was employed, with the assistance of design expert software (Version 12, Srat-Ease Inc., Mineapolis, MN, USA). spheronization time (A), Extruder speed (B), Spheronization speed (C) were selected as independent variables and % yield (R1) was selected as dependent variables based on trial-and-error studies.

The independent and dependent variables are tabulated in table 2.

Table 1: Formulation of pellets of avicel® PH-101 by direct inoculation of *L. casei and L. plantarum* probiotics

Formulation code for *L. casei*	Formulation code for *L. plantarum*	Avicel® PH-101 (g)	Inulin (g)	Pectin (g)	PVP (g)
D1	E1	95	-	-	5
D2	E2	94	1	0	5
D3	E3	94	0	1	5
D4	E4	93	2	0	5
D5	E5	93	0	2	5
D6	E6	93	1	1	5
D7	E7	91	2	2	5
D8	E8	92	1	2	5
D9	E9	92	2	1	5

Table 2: Variables in factorial design for preparation and optimization of directly inoculated pellets

Factors	Levels	
Independent variables	Low	High
A=Spheronization time	2	5
B=Extruder speed	25	31
C=Spheronization speed	1100	1350
Dependent variable	Goals	
Y1=% yield	Increase	

Enteric coating of pellets

The pellets were enteric-coated using Kollicoat® MAE 30DP employing a Mini lab coater (Umang, Mumbai). The enteric coating of Pellets is essential to protect the probiotic pellets from withstanding the harsh GIT condition.

Pellets of 220g were used for the coating. Kollicoat 30 DPI dispersed in water along with propylene glycol as plasticizer of the required amount as described in table 3.

Table 3: Composition of the coating solution

Ingredients	Composition (%)
Kollicoat 30 DPI	50
Propylene glycol	5
Purified Water	Quantity Sufficient

A magnetic stirrer was used to swirl the dispersion for 2 h to obtain a homogeneous dispersion. After that, the coating dispersion was applied to the core pellets maintaining the parameters as mentioned in table 4 in a Mini lab coater (Umang, Mumbai, India).

Table 4: Enteric coating parameters

Parameter	Limits
Air Flow rate (m^3 $h^{-1)}$	95-99
Inlet temperature (°C)	40-42
Atomozing air Pressure (bar)	1.2
Feed spray rate (g min^{-1})	2.1-3

The coated granules were further subjected to characterization and evaluation parameters

Characterization and evaluation of enteric-coated pellets

% Total Yield and % useful yield

The yield was calculated by weighing the pellets and then calculating the percentage yield based on the weight of the input ingredients, which included Avicel 101 and excipients. The size range of 1400–850 µm was chosen as an appropriate size, and palletization yield is reported as the weight of pellets in this range. The following equation 1 and 2 are used for the calculation of % Yield and % useful yield, respectively [22].

$$\% \text{ yield } = \frac{\text{Wt of pellets}}{\text{Wt of Avicel} + \text{Excipients}} \times 100 \ \ldots\ldots (1)$$

$$\% \text{ useful yield } = \frac{\text{Wt of usable pellets}}{\text{Wt of Avicel} + \text{Excipients}} \times 100 \ \ldots\ldots (2)$$

Micromeritic Properties of prepared pellets

The prepared pellets (Formulation D1-D9 and E1-E9) were subjected to micromeritic properties evaluation [23]. The bulk density, tap density (Electro Lab ETD 1020), compressibility index, Hausner's ratio and angle of repose (Eectrolab Manual powder flow tester) were calculated using the formulas (3, 4, 5, 6 and 7) respectively [24].

$$\rho b = \frac{M}{Vb} \ldots\ldots (3)$$

Here, M denotes the mass of powder and Vb denotes the bulk volume of the powder.

$$\rho t = \frac{M}{Vt} \ldots\ldots (4)$$

M-mass of Pellets and Vt-tapped volume of pellets.

$$\% \text{ Compressibility Index } = \frac{\rho\text{tapped} - \rho\text{bulk}}{\rho\text{tapped}} \times 100 \ \ldots\ldots (5)$$

$$\text{Hausner ratio } = \frac{\rho\text{tapped}}{\rho\text{bulk}} \ldots. (6)$$

$$\text{Tan}\theta = \frac{h}{r} \ldots\ldots (7)$$

Here h denotes height, r denotes radius.

Loss on drying

The moisture content was analyzed by placing 5g of sample in a halogen moisture balance analyzer till a constant weight was attained. LoD was calculated using the formula 8.

$$\text{Percentage Loss on drying} = \left(\frac{\text{Initial weight} - \text{Final weight}}{\text{Initial Weight}}\right) \times 100 \ \ldots\ldots (8)$$

Friability

10 g of pellets were weighed and transferred to the friability apparatus (Electrolab, Mumbai) and rotated at 25 RPM for 4 min. These pellets were weighed again after dedusting. The friability was determined as the % loss in weight of the pellets. The experiment was repeated three times for each batch. The friability was calculated using the formula 9.

$$\text{Friability\%} = \frac{\text{Initial Weight} - \text{Final Weight}}{\text{Initial Weight}} \times 100 \ldots\ldots\ldots (9)$$

Scanning electron microscopy (SEM)

SEM (Zeiss EVO LS 15) was employed to determine the pellet's surface morphology; the mounted samples were placed on an aluminium surface using adhesive tape and coated with gold at 70m Torr Pressure for 30u seconds under the influence of vacuum and were scanned at a required magnification at a voltage potential of 15-30 KV prior to observation.

Viability count

Enteric-coated Pellets of 1 g equivalent to uncoated pellets were gently grounded in a mortar and added in 2 ml of pH 6.8 phosphate buffer and further diluted up to 10 ml of MRS broth medium to assess the viable count of *L. casei* and *L. plantarum* present in the pellets [3] respectively. The probiotic-containing broth medium was vortexed for 10-15 seconds and set aside for 2 h at 37 °C in an incubator, after which the suspension was vortexed again for 10 seconds to achieve homogeneity [25]. The broth medium was used to dilute the suspension in a series of steps. The viable count was assessed using the pour plate method with a colony counter after serial dilutions of the probiotic suspension were incubated aerobically at 37 °C for 48 h in MRS agar medium. The pour plate was performed in triplicates [26].

Disintegration time (DT)

Accurately weighed 1g of enteric-coated pellets were placed in the individual basket rack assembly (Electrolab disintegration tester) along with sinkers and the disintegration time of the pellets was determined at 37 °C at a speed of 30 dips/min.

Survivability in Simulated gastrointestinal conditions

Survibility of *L. casei* and *L. plantarum* enteric-coated pellets were analyzed under mimicked gastric environment and intestinal fluids environment using 0.1g of pellets or free cells following the method previously described by *Dimitrellou et al.* [21] with minor modifications.

Simulated gastric juice of 9.9 ml (pH 1.2) and simulated intestinal juices of 9.9 ml (pH 6.8) in a test tube was used to test survivability by maintaining at 37±0.5 °C. For acidic conditions, a 0.5 ml sample was taken after 0, 30, 60, and 120 min, and for bile tolerance, a 0.5 ml sample was taken after 0, 30, 60, 120, and 240 min [27]. The sampled solution was subjected to suitable serial dilution and the count was estimated by pour plate technique using MRS agar media and incubating at 37 °C for 48 h. The test was run in triplicate.

Stability studies

The formulations containing *L. casei* and *L. plantarum* strains that exhibited better viability for each prebiotic ratio were selected to study the impact of prebiotics (inulin and pectin) on viability during the storage conditions. Formulation D1 and E1 prepared without using prebiotics were considered as a control for this stability group.

The stability study was carried out according to ICH guidelines by storing the samples in a well-closed glass bottle and were placed at 25±2 °C, 60%RH, 4±2 °C, and-20±2 °C for a period of 90 d. Samples were analyzed for probiotic cell viability on 0, 15th, 30th, 60th and 90th day.

Statistical analysis

All tests were conducted in triplicates. The significance of the tests carried out was determined at $P<0.05$. The findings were subjected to statistical significance using ANOVA [28], t-test. The significant difference between the results ($P<0.05$) was analyzed using SPSS statistical software (IBM, V. No; 20).

RESULTS AND DISCUSSION

Pre-formulation studies

Fourier transform infrared spectroscopy (FT-IR)

The peaks of corresponding functional groups of pure excipients Avicil-101, Kollicoat, Pectin, and Inulin were compared individually, as a physical mixture and as formulation after enteric coating, as depicted in fig. 1 and table 5. From the results of the FTIR analysis, it was evident that no chemical interactions were observed between excipients used for formulating the pellets.

Differential scanning calorimetry (DSC)

DSC thermograms obtained for pure excipients, physical mixture and formulation are depicted in fig. 2 and table 5. The interpretation of DSC thermograms for pure samples and physical mixtures are listed in table 1. Pectin exhibited multiple peaks, the initial peak around 128 °C was because of the characteristic melting and the peak around 247 °C is because of the charring of the carbohydrate complex in the polymer. The DSC thermogram of pure excipients and physical mixtures did not show any significant shift in the peak of physical mixture and formulation when compared with pure excipients indicating that there were no interactions between the excipients used for the formulation.

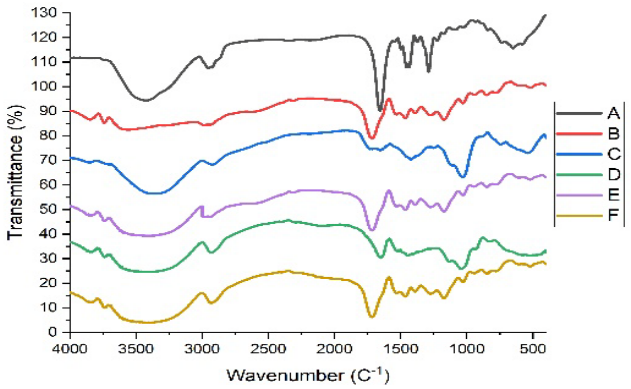

Fig. 1: FT-IR spectrum of samples: A. Avicil-101; B. Kollicoat; C. Pectin D. Inulin; D. Physical mixture and E. Formulation

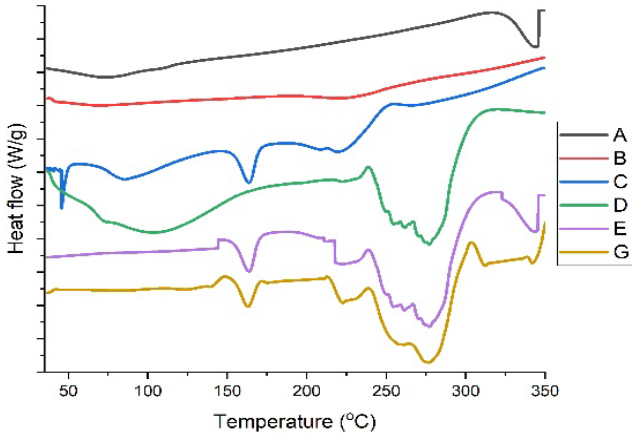

Fig. 2: DSC Thermogram of samples: A. Avicil-101; B. Kollicoat; C. Pectin D. Inulin; D. physical mixture and E. formulation

Table 5: FT-IR and DSC peak interpretation data of Pure samples, physical mixture, and formulation

S. No.	Compound	Spectral characterization			
1.	Avicel® PH-101	IR (KBr): 3358.18 (OH); 2885.39 (CH2OH); 2551.9 (C-O-C); 2142.98 (C-O); 1653.05 (C-O); 1315 (C-C)			
		Onset: 297.62; Peak: 321.94; End peak: 335.21.			
2.	Inulin	IR (KBr): 3853.9(OH); 3749.74(OH); 2929 (C-C); 1433.15 (C-O-C);			
		DSC: Peak No	Onset °C	Peak °C	End set °C
		1	121.11	128.14	136.26
		2	237.61	247.59	244.08
3.	Pectin	IR (KBr): 3860.65 (OH); 3377.46 (OH); 2906.82 (C-O-C); 1665.58 (C-C); 1421.58 (C-C)			
		Onset: 161.35;Peak: 164.94;End peak: 170.62;			
4.	Kollicoat® MAE 30DP	IR (KBr): 3843.29 (OH); 3745.88 (OH); 1726.34 (C=O); 1469.8 (C-C); 1383.97 (C-C)			
		Onset: 208.63;Peak: 222.66;End peak: 241.22			
5.	Physical Mixture	IR (KBr): 3866.43 (OH); 3736.24 (OH); 3551.06 (0H); 2910.67 (C-O-C); 1657.87 (C-C); 1542.13 (C=C); 1464.98 (C-C); 1379.14 (C-C);			
	Avicel 101, Kollicoat MAE 30 DPA, Inulin, Pectin,	DSC Peak No	Onset °C	Peak °C	End set °C
		1	118.82	126.38	134.42
		2	157.35	168.16	175.27
		3	221.91	245.63	249.65
6.	Formulation-D7	3919.79(OH); 3741(OH); 3390(OH); 2912.6(C-O-C); 1652.08(C-C); 1540.21 (C=C),1238.34(C-C);			
		DSC: Peak No	Onset °C	Peak °C	End set °C
		1	112.31	125.26	128.14
		2	155.62	166.63	168.66
		3	220.32	246.45	255.45
		4	315.45	320.86	337.16

Preparation of pellets by extrusion and spheronization technique

Optimization of extrusion spheronization process

Various formulations and process variables that could affect the preparation and properties of pellets were identified and optimized to get small, discrete, and spherical pellets with better process yields. Various parameters like Extruder speed, Spheronizer speed, and Spheronizer time were optimized in this process.

Screening and optimization of process parameters for the preparation of pellets

For the screening of the pellet preparation parameters, 2^3 factorial design was used by keeping spheronization time (A), Extruder speed (B), and Spheronization speed (C), as independent variables. % Yield (R1) as a dependent variable. The 12 trials as suggested by DoE are listed in table 2.

Table 6: Observed response in 2^3 factorial designs for formulation of pellets

Run	Factor 1	Factor 2	Factor 3	Response 1
	A: Time (min)	B: Extrusion speed (RPM)	C: Spheronizer speed (RPM)	R1 % Yield
1	3.5	28	1225	82
2	2	31	1350	92
3	3.5	28	1225	82
4	2	25	1100	84
5	3.5	28	1225	82
6	5	31	1100	74
7	2	31	1100	85
8	5	25	1100	74
9	5	31	1350	74
10	2	25	1350	90
11	3.5	28	1225	77
12	5	25	1350	75

A = Spheronization time (Min), B = Extruder speed (RPM), C = Spheronizer speed (RPM), R1 = % yield

The results as shown in table 2 depicts that the variable chosen had a strong influence on the selected response as the (% yield) was in the range of 74-92 %.

The Model F-value of 23.15 and the ANOVA studies depicted that all models were significant ($p<0.05$) for the selected response parameters. The absence of Fit F-value of 0.82 infers the Lack of Fit was not significant relative to the pure error. Non-significant lack of fit was good for considering a model valid.

The application of factorial design yielded the regression equations as depicted below.

$$R1(\%\ \text{Yield}) = +77.26667 - 4.50000 * \text{Time} + 0.083333 * \text{Extrusion speed} + 0.014000 * \text{Spheronizer speed}$$

Where negative values specify a negative effect of a particular variable on the response factor and positive value specifies the positive effect of a particular variable. The polynomial regression results were expressed by the use of contour plots fig. 3.

The 3D model fig. 3(A) suggested that by decreasing (A) whilst increasing the factors (B) and (C) influenced the % yield positively for all the prepared formulations. From fig. 3(B), it was evident that the predicted vs actual results were well within the acceptable range and were found to be significant as the P-value was<0.05 supported by ANOVA. The counterplot graph fig. 3(C) showed the results obtained at various run conditions. Based on the DoE the various factors finalized were spheronization time-2 Min, Extrusion speed–31 RPM and Extruder speed 1350 RPM.

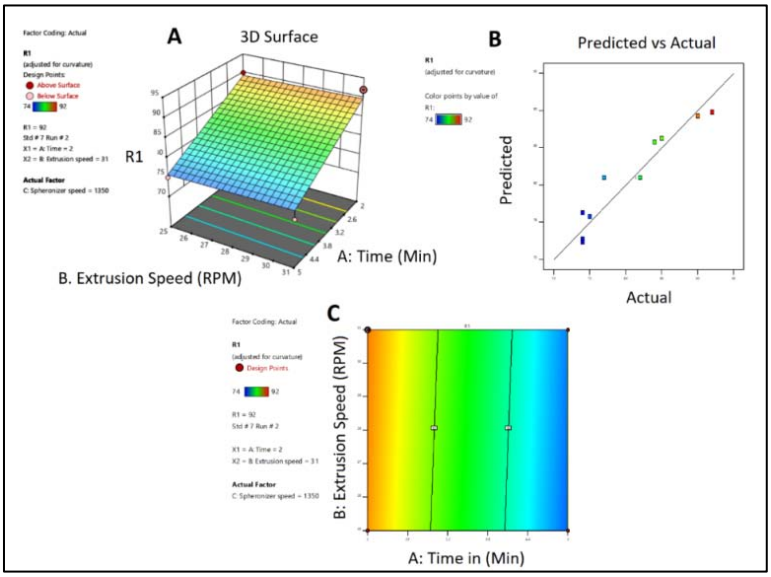

Fig. 3: Graphical representation of the impact of spheronization time, extrusion speed and spheronization speed on % yield, A: 3D Plot analysis; B: Predicted vs Actual results; C: Counter plot

Preparation of directly inoculated pellets of Avicel® PH-101 and *L. casei and L. plantarum* strains

For the preparation of pellets by directly inoculating *L. casei and L. plantarum* strains, various formulations (D1-D9 and E1-E9) as described in the procedural part were carried out. The amount of granulating agent (Water and Skim Milk probiotic dispersion) was in the range of 60-65 ml. The initial concentration of the probiotic concentration in the granulating mass was attuned to contain 9.74 log CFU/g. These pellets were further enteric coated with Kollicoat MAE 30DP employing a fluidized bed processor.

% Total yield and % useful yield

The prepared pellets of *L. casei and L. plantarum* were subjected to various characterization and evaluation parameters. The total % yield ranged from 95.77±0.64 to 92.13±1.92 and 96.17±0.42% to 92.20±1.83%, respectively. The loss of granulated feed by sticking to the extruder and at the time of mixing in RMG and agglomeration during enteric coating resulted in the loss of 5-7%. The % useful yield for *L. casei* enteric-coated pellets was in the range of 87.33±1.24 to 83.94±1.05 and for *L. plantarum* pellets in the range of 87.63±0.89% to 85.40±1.11%. Very few pellets in the % range of 0.73±0.058% to 1.07±0.231% were

retained on sieve #14, which was not considered for the % useful yield. A major portion of pellets (82%) were retained on sieve # 16 to #18, indicating the pellets were in the size range of 1390 µm to 1000 µm. The results of the same are indicated in table 7 and table 8.

Micromeritic properties

The prepared pellets were subjected to various micromeritic properties. *L. casei* loaded enteric-coated pellets displayed Carr's index ranging from 11.53±0.503% to 12.80±0.529 %, Hausner ratio ranged from 1.13±0.008 to 1.15±0.008 and the angle of repose ranged from 31.53 °±0.622 to 33.69 °±0.245 indicating good flow properties (table 7).

The pellets of *L. plantarum* also showed similar micromeritic properties as the car's index ranged from 11.63±0.551 to 12.67±0.577, Hauser's ratio ranged from 1.13±0.006 to 1.15±0.008 and the angle of repose ranged from 31.42±0.679 to 33.52±0.369% indicating good flow properties (table 8).

Loss on drying

Evaluation of pellets for the amount of moisture content present in the pellets of *L casei*, and *L. plantarum* enteric coated pellets after drying was determined in the range of 3.07±0.30% to 2.13±0.11% (table 7) and 2.07±0.11% to 2.84±0.08% (table 8) respectively indicating uniform drying and presence of moisture in the acceptable range.

Friability

The friability results of the prepared pellets of *L casei* and *L. plantarum* enteric-coated pellets were in the range of 0.36±0.148% to 0.23±0.052% (table 7) and 0.20±0.095 to 0.33±0.161 (table 8) well within the acceptable range.

Scanning electron microscopy (SEM)

The analysis of directly inoculated pellets containing Avicel 101, *L. casei and L. plantarum* bacterial dispersion and prebiotics showed the formation of spherical pellets fig. 4 (A, D) with little deformations, some of the bacterial strains on the outer surface of the pellets were seen fig. 4 (C, D) indicating the presence of probiotic strains on the surface of the pellets. The enteric-coated pellets (E) were spherical to oval, with few agglomerations of pellets. The surface of the enteric-coated pellets was smooth and uniform.

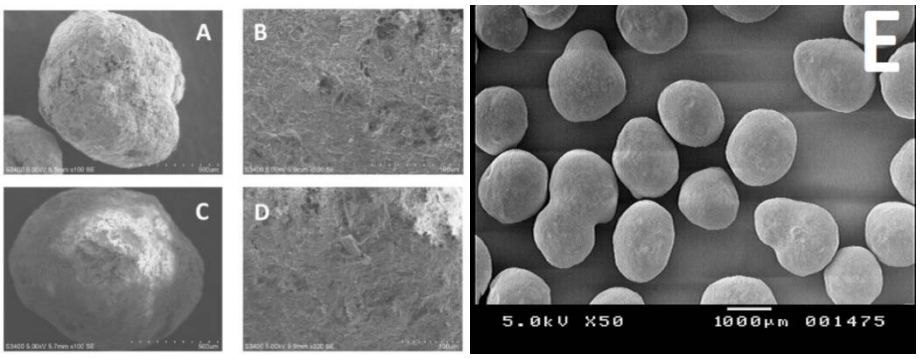

Fig. 4: Morphology of Prepared pellets: A. Formulation D7 pellets of *L. casei* at 100x.; B. Pellets surface morphology at 500X; C. Formulation E5 pellets of *L plantarum* at 100X; D. Pellets surface morphology at 320X; E. Enteric Coated Pellets D7 at 50X

Viability count

Viability count of *L casei* ranged from 8.78±0.31 to 8.53±0.15 log CFU/g and for *L. plantarum* pellets, viability was in the range of 8.47±0.15 to 8.85±0.22 log CFU/g. A log reduction of 0.96 to 1.21 and 0.76 to 1.14 was observed, indicating the impact of drying on the viability of both the bacterial strains. The results are mentioned in table 7 and table 8.

Disintegration time

The disintegration test of the enteric-coated pellets of both *L casei* (D1-D9) *L. plantarum* (E1-E9) revealed that the pellets had an acceptable disintegration time of<15 min. Since Avicel pH 101 was the main pelletizing agent, it was reported in the literature that Avicel has very poor disintegration properties [29], to overcome this issue, polyvinyl pyrrolidone was used as a disintegrating agent, which helped disintegrate the pellets in an acceptable time.

Survivability in simulated gastrointestinal conditions

Survivability in acidic medium

Various studies have proven that a significant loss of viability of probiotic strains is seen when they transit through the low pH of the stomach and the concentrated bile salt GIT conditions. Free cells of *L. casei and L. plantarum* showed a drastic decline in viability from an initial 10.12±0.10 log CFU/ml to 1.58±0.18 log CFU/ml and 9.42±0.14 log CFU/ml to 1.62±0.11 log CFU/ml respectively at the end of 2 h. indicating the necessity for an enteric coating to protect them. The use of the enteric coating in the formulation safeguarded bacterial cells from being exposed to an acidic environment due to the effective coating, resulting in the microorganism's non-release in [21, 30] acidic environment. The enteric polymer and the polymers used to prepare pellets have been used for many tried and tested formulations and are reported to have no impact on the host's normal functioning [31].

Table 7: %Total yield, % Useful yield, Cars index, Hausner ratio, Angle of repose, LoD, % Friability and viability of enteric-coated Pellets *L. casei*

Formulation	% Total yield ±SD*	% Useful yield ±SD*	Carr's index (%)±SD*	Hausner ratio±SD*	Angle of repose θ °±SD*	%LOD±SD*	% Friability±SD*	Viability log CFU/g±SD*
D1	95.77±0.64	86.33±1.10	11.73±0.643	1.13±0.008	33.69±0.245	3.07±0.30	0.23±0.052	8.58±0.22
D2	93.30±0.75	86.90±1.23	12.50±0.500	1.14±0.007	33.30±0.477	2.58±0.37	0.24±0.010	8.78±0.31
D3	93.83±1.26	83.94±1.05	12.67±0.577	1.15±0.008	33.18±0.686	2.52±0.28	0.26±0.111	8.68±0.12
D4	92.57±1.78	86.47±0.98	12.80±0.529	1.15±0.007	33.62±0.161	3.03±0.27	0.36±0.054	8.63±0.23
D5	93.83±1.50	86.83±1.12	11.53±0.503	1.13±0.006	33.27±0.526	2.46±0.29	0.36±0.055	8.53±0.15
D6	93.17±1.80	87.33±1.24	11.67±0.577	1.13±0.007	33.68±0.242	2.13±0.11	0.30±0.167	8.73±0.18
D7	91.90±1.21	86.73±1.31	12.37±0.635	1.14±0.008	31.53±0.622	2.20±0.19	0.36±0.148	8.69±0.21
D8	93.67±1.03	86.53±1.02	12.63±0.569	1.14±0.007	33.62±0.212	2.38±0.38	0.33±0.203	8.56±0.26
D9	92.13±1.92	86.33±0.89	12.33±0.577	1.14±0.008	33.10±0.547	2.39±0.33	0.33±0.029	8.71±0.18

*Standard Deviation n=3

Table 8: %Total yield, % useful yield, cars index, hausner ratio, angle of repose, LoD, % friability and viability of enteric-coated pellets of *L. plantarum*

Formulation	% Total yield ±SD*	% Useful yield ±SD*	Carr's index(%)±SD*	Hausner ratio±SD*	Angle of repose θ±SD*	%LoD ±SD*	% Friability ±SD*	Viability log CFU/g±SD*
E1	96.17±0.42	87.63±0.89	11.93±0.306	1.14±0.004	33.27±0.627	2.13±0.11	0.20±0.095	8.45±0.15
E2	93.49±1.19	87.57±1.04	12.60±0.422	1.15±0.007	33.52±0.369	2.83±0.38	0.27±0.049	8.68±0.21
E3	93.63±1.23	86.73±1.20	12.53±0.503	1.14±0.007	31.42±0.679	2.84±0.08	0.26±0.047	8.53±0.12
E4	92.67±0.81	86.83±0.95	12.60±0.529	1.14±0.007	32.67±0.680	2.33±0.40	0.28±0.006	8.57±0.16
E5	94.23±1.19	86.43±1.33	12.61±0.693	1.14±0.009	33.43±0.663	2.07±0.11	0.23±0.058	8.47±0.31
E6	92.27±0.95	86.57±0.78	11.68±0.563	1.13±0.007	33.02±0.575	2.20±0.19	0.30±0.094	8.76±0.24
E7	92.20±1.83	85.97±1.10	11.83±0.764	1.13±0.010	33.12±0.239	2.39±0.33	0.32±0.171	8.78±0.22
E8	92.80±1.97	85.93±0.85	11.63±0.551	1.13±0.006	33.07±0.543	2.33±0.11	0.33±0.161	8.55±0.30
E9	92.43±2.30	85.40±1.11	12.67±0.577	1.15±0.008	33.44±0.242	2.53±0.29	0.32±0.210	8.68±0.21

*Standard Deviation n=3

Survivability in bile medium

The impact of bile salts on the viability of *L. casei* and *L. plantarum* bacterial strains was determined in this study by using free cell as control, the results indicated that the cells were susceptible to bile salts too, but not to the extent as acidic environment.

The initial viability of Free cell of *L. casei* and *L. plantarum* was 10.12±0.10 log CFU/ml and 9.42±0.11 log CFU/ml and at the end of 3h the cell viability was 2.14±0.26 log CFU/ml and 1.8±0.21 log CFU/ml respectively. A reduction of 78% and 81% was observed, indicating the impact of bile salts on long exposure. The formulations D1 and E1 did not contain any prebiotic in their core matrix and showed less viability at the end of 3 h in comparison to all other formulations, which contained inulin and pectin as prebiotics. This indicated that prebiotics had a positive impact on protecting the cell in the bile environment. D2 and E2 containing (1%) inulin showed 4.59±0.15 log CFU/g and 4.54±0.22 log CFU/g, an increase of 1.67% and 3.02% increase in viability was observed as the concentration of inulin increased from 1% to 2% in formulations E3 and D3 respectively. Formulation D4 and E4 containing pectin (1%) showed 4.68±0.21 log CFU/ml and4.75±0.17 log CFU/ml. Formulations D5 and E5 pectin (2%) had 4.95±0.20 log CFU/g and 5.05±0.11 log CFU/g.

Formulations D6 to D9 and E6 to E9 were studied to understand the impact of the combination of inulin and pectin [32] on the viability, the presence of prebiotics showed a better effect when used in combination. All these formulations showed higher viability in comparison to the individually used prebiotic formulations. Within the combination used, formulations D7 and E7 containing 2% inulin and pectin showed the highest viability of 56.22% and 58.32% viability respectively in comparison to the initial cell count. This indicated that increasing the concentration of prebiotics improved the viability in the presence of GIT conditions. The dissolution studies results are graphically represented in fig. 5.

Subjecting the results to one-way ANOVA analysis revealed the results were not significant P>0.005 in both the strain formulations, as there was no major difference in viability among the formulations when compared within the group. On the statistical evaluation of the viability of the formulations, which showed better viability and free cell with a paired t-test, a significant improvement was observed in formulation D7 and free *L. casei* strains (P<0.005). Similarly, a significant improvement was observed in *L. plantarum* formulation E7, E8, and E9 and free *L. plantarum* strains (P<0.005).

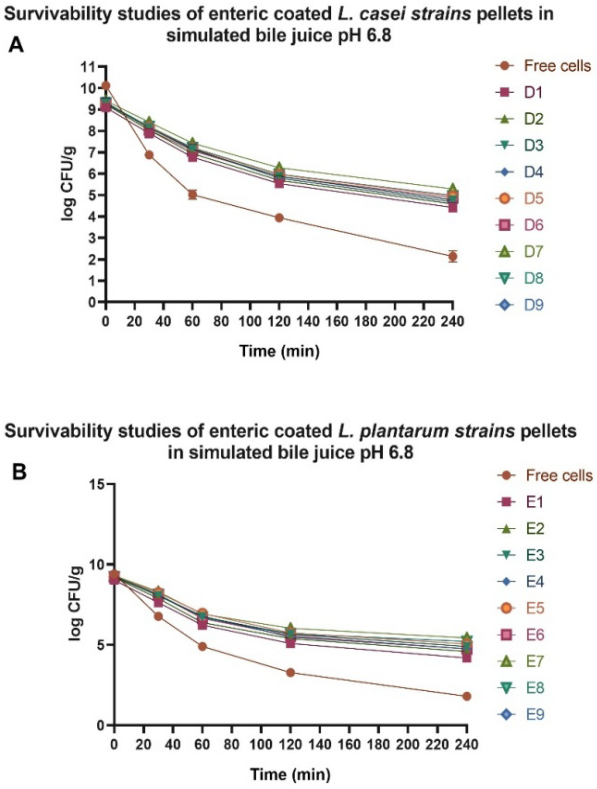

Fig. 5: Survivability studies of enteric-coated pellets in simulated bile juice pH 6.8: A. L. Casei strains pellets B. L. Plantarum strains

Prebiotics are supplements that have a positive impact on health by selectively promoting the growth of lactobacilli species [33], because of their chemical properties they are not digestible in the small intestine but act as a metabolic energy source for the colon microbes. Studies have shown that pectin and inulin are the most reliable prebiotics, as they also have additional benefits by resisting gastric and GIT enzymes in the laboratory and real-world conditions [34]. Nazzaro *et al.* l [17]., have reported that *L. casei* and *L. plantarum* showed a good proliferation rate when grown in the presence of inulin and pectin as a substrate. Pectin and a few oligosaccharides have been employed to formulate targeted drug delivery in a few drug dosage forms [35, 36] but are to be explored to be used to target probiotic release. This fact supports the improved survival of strains in the current study in the presence of inulin and pectin in comparison to free cells, as they resist degradation when it passes through gastric environment and is selectively fermented in the location of the colon. Increasing the ratio of Inulin and pectin from 1% to 2 % showed better viability in the bile conditions since the pectin matrix exhibited gelation property on exposure to water content resulting in slower exposure of bacterial strains to harsh conditions and in turn, improving the viability [37].

Stability studies

Stability studies of enteric-coated *L. casei* and *L. Plantarum* pellet formulations

The formulations which showed better survivability in testing using inulin and pectin individually and in combination were subjected to stability studies for 3 mo under three different conditions i. e 25±2 °C, 4±2 °C and-20±2 °C. Stability data of formulations stored at 25±2 °C as mentioned in table 9 and table 10 revealed that formulations D7 and E7 showed the highest viability of 8.35±0.19 log CFU/g and 8.14±0.23 log CFU/g at the end of 3 mo, other enteric-coated formulations also qualify for the minimum probiotic concentration i. e 6 log CFU/g, but formulation D1 and E1 which was not containing any prebiotic in its core matrix had the least viability at the end of 3 mo. The formulations stored at 4±2 °C showed better viability in comparison to the previous storage conditions, indicating that storing the formulations under refrigerator conditions improved the viability. Among the four formulations, D7 and E7 showed viability of 8.4±0.19 log CFU/g and.20±0.22 log CFU/g. Formulation D1 and E1 had the least viability of 6.38±0.22 log CFU/g and 7.47±0.20 log CFU/g. the data is mentioned in table 9 and table 10. The data from the storage temperature of-20±2 °C as mentioned in table 9 and table 10 revealed that the formulations viability improved as the temperature of the storage condition decreased. D7 and E7 showed viability at 8.53±0.14 log CFU/g and 8.21±0.13 log CFU/g, respectively. The formulations D1 and E1, which did not have prebiotics showed the viability of 6.15±0.19 log CFU/g and 7.99±0.14 log CFU/g, qualifying for the minimum quantity of bacteria required for a probiotic formulation.

Storage temperature and time affect the survival of bacteria in the formulations [38]. Thus, understanding the suitable storage conditions to improve viability becomes important [39]. Bacteria are very susceptible to high temperatures generally hence refrigeration conditions are reported optimum for probiotic storage [40]. Enteric coating [11] has helped in reducing the exposure of bacteria directly to atmospheric and oxygen conditions and thereby improving the viability in comparison to non-enteric coated pellets in all the storage conditions [41]. One way ANOVA analysis of all the stability data within groups did not show any significance; all the formulations had P>0.005, indicating that the storage temperatures among the selected temperature did not have a greater impact on storage and providing supporting evidence that all the developed formulations containing prebiotics influenced the viability of probiotics on in the developed formulations.

Table 9: Stability studies for enteric-coated L. casei pellets at 25±2 °C, 60%±5% RH; 4±2 °C and -20±2 °C

Storage conditions	Formulation	Viable counts (log CFU/g)				
		0th day	15th day	30th day	60th day	90th day
25±2 °C 60%±5% RH	D1	9.31±0.21	8.54±0.18	7.86±0.11	6.84±0.12	6.31±0.23
	D3	9.34±0.15	9.04±0.14	8.85±0.20	8.47±0.16	8.01±0.16
	D5	9.28±0.20	9.00±016	8.89±0.16	8.50±0.15	8.05±0.11
	D7	9.41±0.14	9.21±0.21	9.03±0.19	8.77±0.22	8.35±0.19
4±2 °C	D1	9.31±0.20	8.41±0.23	7.89±0.15	6.98±0.18	6.38±0.22
	D3	9.34±0.15	9.09±0.13	8.91±0.19	8.55±0.15	8.04±0.15
	D5	9.28±0.19	9.08±0.20	8.88±0. 23	8.67±0.20	8.17±0.11
	D7	9.41±0.16	9.23±0.14	9.06±0.15	8.81±0.11	8.40±0.19
-20±2 °C	D1	9.31±0.22	8.51±0.12	7.83±0.17	6.92±0.13	6.15±0.19
	D3	9.34±0.10	9.13±0.15	8.94±0.18	8.50±0.22	8.18±0.11
	D5	9.28±0.14	9.06±0.21	8.86±0.13	8.44±0.20	8.21±0.21
	D7	9.41±0.20	9.21±0.14	9.03±0.16	8.64±0.11	8.53±0.14

Results expressed in mean±SD, n=3

Table 10: Stability studies for enteric-coated *L. plantarum* pellets at 25±2 °C, 60%±5% RH; 4±2 °C and-20±2 °C

Storage conditions	Formulation	Viable counts (log CFU/g)				
		0th day	15th day	30th day	60th day	90th day
25±2 °C 60%±5% RH	E1	9.28±0.14	8.46±0.16	8.22±0.15	7.68±0.12	7.13±0.13
	E3	9.28±0.15	9.06±0.11	8.83±0.22	8.57±0.23	8.06±0.15
	E5	9.24±0.20	9.01±0.13	8.80±0.26	8.57±0.19	7.98±0.11
	E7	9.31±0.16	9.13±0.19	8.87±0.14	8.65±0.15	8.14±0.23
4±2 °C	E1	9.28±0.21	8.50±0.23	8.25±0.21	8.03±0.11	7.47±0.20
	E3	9.28±0.16	9.10±0.21	8.88±0.16	8.69±0.21	8.18±0.13
	E5	9.24±0.21	9.03±0.18	8.79±0.17	8.61±0.19	8.08±0.18
	E7	9.31±0.11	9.11±0.15	8.89±0.19	8.70±0.17	8.20±0.22
-20±2 °C	E1	9.28±0.23	9.04±0.15	8.8±0.17	8.57±0.11	7.99±0.14
	E3	9.28±0.17	9.06±0.19	8.83±0.14	8.63±0.23	8.09±0.19
	E5	9.24±0.11	9.03±0.11	8.81±0.16	8.60±0.19	8.08±0.21
	E7	9.31±0.20	9.11±0.15	8.90±0.20	8.71±0.12	8.21±0.13

Results expressed in mean±SD, n=3

CONCLUSION

It was concluded from the present work that the probiotic *L. casei* and *L. plantarum* could be incorporated into pellets avoiding the high heating temperatures and complex procedures like freeze-drying.

The concept of enteric coating offers the potentially increased efficacy of survival of the probiotics. The tolerance to an acidic environment and the improved viability of probiotics along with prebiotics such as inulin and pectin was found to be concentration-dependent. The good viability of probiotics during the stability conditions also provides support to explore the formulation of probiotics as pellets. The incorporation of prebiotics also has a beneficial effect on the viability of probiotics in long-term storage. Hence this study suggested that the usage of extrusion and spheronization process and prebiotics (Inulin and Pectin) can be utilized to formulate probiotic dosage forms with improved viability in physiological conditions and real-time storage conditions.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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